UTILITY PATENT APPLICATION TRANSMITTAL *(Only for new nonprovisional applications under 37 CFR 1.53(b))*	*Attorney Docket No.* 00263/PP/IR/7644 US-DIV-2 — *Total Pages :*
	First Named Inventor or Application Identifier Edwin Southern
	Express Mail Label No.:

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
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1. [X] **Fee Transmittal Form**
   (Submit an original, and a duplicate for fee processing)

2. [X] **Specification** *[Total Pages -32]*
   (preferred arrangement set forth below)
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D

   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure

3. [] **Drawing(s)** *(35 USC 113)* *[Total sheets -]*
4. [X] **Oath or Declaration** *[Total Pages - 2]*
   a.1. [] Newly executed (original or copy)
   a.2. [] Unexecuted
   b. [X] Copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 17 completed)
   [Note Box 5 below]
   i. [] <u>DELETION OF INVENTOR(S)</u>
      Signed statement attached deleting inventor(s)
      named in the prior application, see 37 CFR
      1.63(d)(2) and 1.33(b).

5. [X] **Incorporation By Reference**
   (usable if Box 4b is checked)
   The entire disclosure of the prior application, from which
   a copy of the oath or declaration is supplied under Box
   4b, is considered as being part of the disclosure of the
   accompanying application and is hereby incorporated by
   reference therein.

6. [] Microfiche Computer Program *(Appendix)*

7. [] Nucleotide and/or Amino Acid Sequence Submission
   (if applicable, all necessary)
   a. [] Computer Readable Copy
   b. [] Paper Copy (identical to computer copy)
   c. [] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [] Assignment Papers (cover sheet & document(s))
9. [] 37 CFR 3.73(b) Statement [] Power of Attorney
   (when there is an assignee)
10. [] English Translation Document *(if applicable)*
11. [X] Information Disclosure Statement (IDS)/PTO-1449
    [] Copies of IDS Citations
12. [X] Preliminary Amendment
13. [X] Return Receipt Postcard (MPEP 503)
    (Should be specifically itemized)
14. [] Small Entity Statement(s)
    [] Statement filed in prior application, Status still proper and desired
15. [] Certified Copy of Priority Document(s)
    (if foreign priority is claimed)
16. [X] Other   CLAIM OF PRIORITY

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*
    [] Continuation   [X] Divisional   [] Continuation-in-part (CIP) of prior Application No. 08/925,676

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October 22, 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of   :

Edwin Southern   :

Serial No. NEW   :   **Attn: Application Branch**

Filed October 22, 1999   :   **Attorney Docket No.**
                                   00263/PP/IR/7644 US-DIV-2

ANALYSING POLYNUCLEOTIDE SEQUENCES
(Rule 1.53(b) Divisional of Serial No. 08/925,676,
Filed September 9, 1997)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
    Washington, DC 20231

Sir:

    Kindly amend the application as follows:

IN THE SPECIFICATION

    Page 1, lines 1 and 2 (after the title), please rewrite the sentence as follows: -- This is a divisional application of S.N. 08/925,676 filed September 9, 1997, which is a divisional of application S.N. 08/230,012 filed April 19, 1994, now U.S. Patent No. 5,700,637, which is a continuation of abandoned application S.N. 08/695,682 filed May 3, 1991, which is a continuation-in-part of abandoned application S.N. 07/573,317 filed September 28, 1990, which is a 371 of international application PCT/GB89/00460 filed May 2, 1989. --

IN THE CLAIMS

    Please cancel claims 2-16 without prejudice.

IN THE ABSTRACT

Please insert the Abstract which is attached hereto as a single sheet at the beginning of the application papers.

REMARKS

This application is a divisional of S.N. 08/925,676. The application is intended to be directed to certain claims which were canceled without prejudice in the parent application. Applicant intends to file a preliminary paper formally presenting such claims prior to issuance of the first Action.

Favorable action is respectfully solicited.

Respectfully submitted,

Edwin Southern

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October 22, 1999

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ANALYSING POLYNUCLEOTIDE SEQUENCES

This is a CIP of Serial No. 07/573,317 filed
September 28, 1990.

1.   INTRODUCTION

Three methods dominate molecular analysis of
nucleic acid sequences: gel electrophoresis of
restriction fragments, molecular hybridisation, and the
rapid DNA sequencing methods. These three methods have
5   a very wide range of applications in biology, both in
basic studies, and in the applied areas of the subject
such as medicine and agriculture. Some idea of the
scale on which the methods are now used is given by the
rate of accumulation of DNA sequences, which is now
10   well over one million base pairs a year. However,
powerful as they are, they have their limitations. The
restriction fragment and hybridisation methods give a
coarse analysis of an extensive region, but are rapid;
sequence analysis gives the ultimate resolution, but it
15   is slow, analysing only a short stretch at a time. ..
There is a need for methods which are faster than the
present methods, and in particular for methods which
cover a large amount of sequence in each analysis.

This invention provides a new approach which
20   produces both a fingerprint and a partial or complete
sequence in a single analysis, and may be used directly
with complex DNAs and populations of RNA without the
need for cloning.

In one aspect the invention provides
25   apparatus for analysing a polynucleotide sequence,
comprising a support and attached to a surface therof
an array of the whole or a chosen part of a complete
set of oligo nucleotides of chosen lengths, the
different oligonucleotides occupying separate cells of
30   the array and being capable of taking part in
hybridisation reactions. For studying differences
between polynucleotide sequences, the invention
provides in another aspect apparatus comprising a
support and attached to a surface therof an array of
35   the whole or a chosen part of a complete set of
oligonucleotides of chosen lengths comprising the
polynucleotide sequences, the different

oligonucleotides occupying separate cells of the array
and being capable of taking part in hybridisation
reactions.

In another aspect, the invention provides a
5   method of analysing a polynucleotide sequence, by the
use of a support to the surface of which is attached an
array of the whole or a chosen part of a complete set
of oligo nucleotides of chosen lengths, the different
oligonucleotides occupying separate cells of the array,
10 which method comprises labelling the polynucleotide
sequence or fragments thereof to form labelled
material, applying the labelled material under
hybridisation conditions to the array, and observing
the location of the label on the surface associated
15 with particular members of the set of oligonucleotides.

The idea of the invention is thus to provide
a structured array of the whole or a chosen part of a
complete set of oligonucleotides of one or several
chosen lengths. The array, which may be laid out on a
20 supporting film or glass plate, forms the target for a
hybridisation reaction. The chosen conditions of
hybridisation and the length of the oligonucleotides
must at all events be sufficient for the available
equipment to be able to discriminate between exactly
25 matched and mismatched oligonucleotides. In the
hybridisation reaction, the array is explored by a
labelled probe, which may comprise oligomers of the
chosen length or longer polynucleotide sequences or
fragments, and whose nature depends on the particular
30 application. For example, the probe may comprise
labelled sequences amplified from genomic DNA by the
polymerase chain reaction, or a mRNA population, or a
complete set of oligonucleotides from a complex
sequence such as an entire genome. The end result is a
35 set of filled cells corresponding to the
oligonucleotides present in the analysed sequence, and
a set of "empty" sites corresponding to the sequences

which are absent in the analysed sequence. The pattern
produces a fingerprint representing all of the sequence
analysed. In addition, it is possible to assemble most
or all of the sequence analysed if an oligonucleotide

5   length is chosen such that most or all oligonucleotide
sequences occur only once.

The number, the length and the sequences of the
oligonucleotides present in the array "lookup table"
also depend on the application. The array may include

10 all possible oligonucleotides of the chosen length, as
would be required if there was no sequence information
on the sequence to be analysed. In this case, the
preferred length of oligonucleotide used depends on the
length of the sequence to be analysed, and is such that

15 there is likely to be only one copy of any particular
oligomer in the sequence to be analysed. Such arrays
are large. If there is any information available on
the sequence to be analysed, the array may be a
selected subset. For the analysis of a sequence which

20 is known, the size of the array is of the same order as
length of the sequence, and for many applications, such
as the analysis of a gene for mutations, it can be
quite small. These factors are discussed in detail in
what follows.

25 2. OLIGONUCLEOTIDES AS SEQUENCE PROBES

Oligonucleotides form base paired duplexes with
oligonucleotides which have the complementary base
sequence. The stability of the duplex is dependent on
the length of the oligonucleotides and on base

30 composition. Effects of base composition on duplex
stability can be greatly reduced by the presence of
high concentrations of quaternary or tertiary amines.
However, there is a strong effect of mismatches in the
oligonucleotides duplex on the thermal stability of the

35 hybrid, and it is this which makes the technique of

hybridisation with oligonucleotides such a powerful method for the analysis of mutations, and for the selection of specific sequences for amplification by DNA polymerase chain reaction. The position of the
5 mismatch affects the degree of destabilisation. Mismatches in the centre of the duplex may cause a lowering of the Tm by 10°C compared with 1°C for a terminal mismatch. There is then a range of discriminating power depending on the position of
10 mismatch, which has implications for the method described here. There are ways of improving the discriminating power, for example by carrying out hybridisation close to the Tm of the duplex to reduce the rate of formation of mismatched duplexes, and by
15 increasing the length of oligonucleotide beyond what is required for unique representation. A way of doing this systematically is discussed.

3.   ANALYSIS OF A PREDETERMINED SEQUENCE

One of the most powerful uses of oligonucleotide
20 probes has been in the detection of single base changes in human genes. The first example was the detection of the single base change in the betaglobin gene which leads to sickle cell disease. There is a need to extend this approach to genes in which there may be a
25 number of different mutations leading to the same phenotype, for example the DMD gene and the HPRT gene, and to find an efficient way of scanning the human genome for mutations in regions which have been shown by linkage analysis to contain a disease locus for
30 example Huntington's disease and Cystic Fibrosis. Any known sequence can be represented completely as a set of overlapping oligonucleotides. The size of the set is N s + 1 $\approx$ N, where N is the length of the sequence and s is the length of an oligomer. A gene of 1 kb for
35 example. may be divided into an overlapping set of

around one thousand oligonucleotides of any chosen
length. An array constructed with each of these
oligonucleotides in a separate cell can be used as a
multiple hybridisation probe to examine the homologous

5   sequence in any context, a single-copy gene in the human
genome or a messenger RNA among a mixed RNA population,
for example. The length s may be chosen such that
there is only a small probability that any oligomer in
the sequence is represented elsewhere in the sequence

10   to be analysed. This can be estimated from the
expression given in the section discussing statistics
below. For a less complete analysis it would be
possible to reduce the size of the array e.g. by a
factor of up to 5 by representing the sequence in a

15   partly or non-overlapping set. The advantage of using
a completely overlapping set is that it provides a more
precise location of any sequence difference, as the
mismatch will scan in s consecutive oligonucleotides.

4.   ANALYSIS OF AN UNDETERMINED SEQUENCE

20   The genomes of all free living organisms are
larger than a million base pairs and none has yet been
sequenced completely. Restriction site mapping reveals
only a small part of the sequence, and can detect only
a small portion of mutations when used to compare two

25   genomes. More efficient methods for analysing complex
sequences are needed to bring the full power of
molecular genetics to bear on the many biological
problems for which there is no direct access to the
gene or genes involved. In many cases, the full

30   sequence of the nucleic acids need not be determined;
the important sequences are those which differ between
two nucleic acids. To give three examples: the DNA
sequences which are different between a wild type
organism and one which carries a mutant can lead the

35   way to isolation of the relevant gene; similarly, the
sequence differences between a cancer cell and its

normal counterpart can reveal the cause of transfor-
mation; and the RNA sequences which differ between two
cell types point to the functions which distinguish
them. These problems can be opened to molecular
5   analysis by a method which identifies sequence
differences. Using the approach outlined here, such
differences can be revealed by hybridising the two
nucleic acids, for example the genomic DNA of the two
genotypes, or the mRNA populations of two cell types to
10   an array of oligonucleotides which represent all
possible sequences. Positions in the array which are
occupied by one sequence but not by the other show
differences in two sequences. This gives the sequence
information needed to synthesise probes which can then
15   be used to isolate clones of the sequence involved.

4.1   ASSEMBLING THE SEQUENCE INFORMATION

Sequences can be reconstructed by examining the
result of hybridisation to an array. Any oligonucleo-
tide of length s from within a long sequence, overlaps
20   with two others over a length s-1. Starting from each
positive oligonucleotide, the array may be examined for
the four oligonucleotides to the left and the four to
the right that can overlap with a one base displacement.
If only one of these four oligonucleotides is found to
25   be positive to the right, then the overlap and the
additional base to the right determine s bases in the
unknown sequence. The process is repeated in both
directions, seeking unique matches with other positive
oligonucleotides in the array. Each unique match adds
30   a base to the reconstructed sequence.

4.2   SOME STATISTICS

Any sequence of length N can be broken down to a
set of ~ N overlapping sequences s base pairs in
length. (For double stranded nucleic acids, the
35   sequence complexity of a sequence of N base pairs is
2N, because the two strands have different sequences.

but for the present purpose, this factor of two is not
significant). For oligonucleotides of length s, there
are 4^s different sequence combinations. How big should
s be to ensure that most oligonucleotides will be
5   represented only once in the sequence to be analysed,
of complexity N? For a random sequence the expected
number of s-mers which will be present in more than one
copy is

$$\mu_{>1} \approx 4'(1 - e^{-\lambda}(1 + \lambda))$$

10   **where**

$$\lambda = (N - s + 1)/4'$$

For practical reasons it is also useful to know
how many sequences are related to any given s-mer by a
15 single base change. Each position can be substituted
by one of three bases, there are therefore 3s sequences
related to an individual s-mer by a single base change,
and the probability that any s-mer in a sequence of N
bases is related to any other s-mer in that sequence
20 allowing one substitution is 3s x $N/4^s$. The relative
signals of matched and mismatched sequences will then
depend on how good the hybridisation conditions are in
distinguishing a perfect match from one which differ by
a single base. (If 4^s is an order of magnitude greater
25 than N, there should only be a few, 3s/10, related to
any oligonucleotide by one base change.) The
indications are that the yield of hybrid from the mis-
matched sequence is a fraction of that formed by the
perfect duplex.
30   For what follows, it is assumed that conditions
can be found which allow oligonucleotides which have
complements in the probe to be distinguished from those
which do not.
4.3 ARRAY FORMAT, CONSTRUCTION AND SIZE
35   To form an idea of the scale of the arrays needed

to analyse sequences of different complexity it is
convenient to think of the array as a square matrix.
All sequences of a given length can be represented just
once in a matrix constructed by drawing four rows

5 representing the four bases, followed by four similar
columns. This produces a 4 x 4 matrix in which each of
the 16 squares represents one of the 16 doublets. Four
similar matrices, but one quarter the size, are then
drawn within each of the original squares. This

10 produces a 16 x 16 matrix containing all 256 tetra-
nucleotide sequences. Repeating this process produces
a matrix of any chosen depth, s, with a number of cells
equal to 4^s. As discussed above, the choice of s is of
great importance, as it determines the complexity of

15 the sequence representation. As discussed below, s
also determines the size of the matrix constructed,
which must be very big for complex genomes. Finally,
the length of the oligonucleotides determines the
hybridisation conditions and their discriminating power

20 as hybridisation probes.

s	4^s	Genomes	Side of Matrix (pixel=100 μm)	Number of Sheets of film
8	65536	$4^s \times 10$		
9	262144			
10	1.0×10^6	cosmid	100 mm	1
11	4.2×10^6			
12	1.7×10^7			
13	6.7×10^7	E.coli		
14	2.6×10^8	yeast	1.6 m	9
15	1.1×10^9			
16	4.2×10^9			
17	1.7×10^{10}			
18	6.7×10^{10}	human	25 m	2,500
19	2.7×10^{11}			
20	1.1×10^{12}		100 m	

The table shows the expected scale of the arrays
needed to perform the first analysis of a few genomes.
The examples were chosen because they are genomes which
have either been sequenced by conventional procedures -
the cosmid scale -, are in the process of being
sequenced - the E. coli scale -, or for which there has been
considerable discussion of the magnitude of the problem
- the human scale. the table shows that the expected
scale of the matrix approach is only a small fraction
of the conventional approach. This is readily seen in
the area of X-ray film that would be consumed. It is
also evident that the time taken for the analysis would
be only a small fraction of that needed for gel
methods. The "Genomes" column shows the length of
random sequence which would fill about 5% of cells in
the matrix. This has been determined to be the optimum
condition for the first step in the sequencing strategy
discussed below. At this size, a high proportion of
the positive signals would represent single occurrences
of each oligomer, the conditions needed to compare two
genomes for sequence differences.

5.   REFINEMENT OF AN INCOMPLETE SEQUENCE

Reconstruction of a complex sequence produces a
result in which the reconstructed sequence is
interrupted at any point where an oligomer that is
repeated in the sequence occurs. Some repeats are
present as components of long repeating structures
which form part of the structural organisation of the
DNA, dispersed and tandum repeats in human DNA for
example. But when the length of oligonucleotide used
in the matrix is smaller than that needed to give
totally unique sequence representation, repeats occur
by chance. Such repeats are likely to be isolated.
That is, the sequences surrounding the repeated
oligomers are unrelated to each other. The gaps caused

by these repeats can be removed by extending the
sequence to longer oligomers. In principle, those
sequences shown to be repeated by the first analysis.
using an array representation of all possible
5   oligomers, could be resynthesised with an extension at
each end. For each repeated oligomer, there would be 4
x 4 = 16 oligomers in the new matrix. The
hybridisation analysis would now be repeated until the
sequence was complete. In practice, because the
10   results of a positive signal in the hybridisation may
be ambiguous, it may be better to adopt a refinement of
the first result by extending all sequences which did
not give a clear negative result in the first analysis.
An advantage of this approach is that extending the
15   sequence brings mismatches which are close to the ends
in the shorter oligomer, closer to the centre in the
extended oligomer, increasing the discriminatory power
of duplex formation.

5.1   A HYPOTHETICAL ANALYSIS OF THE SEQUENCE OF
20   BACTERIOPHAGE λ DNA

Lambda phage DNA is 48,502 base pairs long. Its
sequence has been completely determined, we have
treated one strand of this as a test case in a computer
simulation of the analysis. The table shows that the
25   appropriate size of oligomer to use for a sequence of
this complexity is the 10-mer. With a matrix of 10-
mers, the size was 1024 lines square. After
"hybridisation" of the lambda 10-mers in the computer,
46,377 cells were positive, 1957 had double
30   occurrences, 75 triple occurrences, and three quadruple
occurrences. These 46,377 positive cells represented
known sequences, determined from their position in the
matrix. Each was extended by four x one base at the 3'
end and four x one base at the 5', end to give 16 x
35   46,377 = 742,032 cells. This extended set reduced the

number of double occurrences to 161, a further 16-
fold extension brought the number down to 10, and one
more provided a completely overlapped result. Of
course, the same end result of a fully overlapped

5   sequence could be achieved starting with a 4^{16} matrix,
but the matrix would be 4000 times bigger than the
matrix needed to represent all 10-mers, and most of the
sequence represented on it would be redundant.

5.2 LAYING DOWN THE MATRIX

10   The method described here envisages that the
matrix will be produced by synthesising oligonucleotides
in the cells of an array by laying down the precursors
for the four bases in a predetermined pattern, an
example of which is described above. Automatic

15   equipment for applying the precursors has yet to be
developed, but there are obvious possibilites; it
should not be difficult to adapt a pen plotter or other
computer-controlled printing device to the purpose.
The smaller the pixel size of the array the better, as

20   complex genomes need very large numbers of cells.
However, there are limits to how small these can be
made. 100 microns would be a fairly comfortable upper
limit, but could probably not be achieved on paper for
reasons of texture and diffusion. On a smooth

25   impermeable surface, such as glass, it may be possible
to achieve a resolution of around 10 microns, for
example by using a laser typesetter to preform a
solvent repellant grid, and building the oligonucleo-
tides in the exposed regions. One attractive

30   possibility, which allows adaptation of present
techniques of oligonucleotide synthesis, is to sinter
microporous glass in microscopic patches onto the
surface of a glass plate. Laying down very large
number of lines or dots could take a long time, if the

35   printing mechanism were slow. However, a low cost ink-

jet printer can print at speeds of about 10,000 spots per second. With this sort of speed, 10^8 spots could be printed in about three hours.

5 5.3 OLIGONUCLEOTIDE SYNTHESIS

There are several methods of synthesising oligonucleotides. Most methods in current use attach the nucleotides to a solid support of controlled pore size glass (CPG) and are suitable for adaptation to

10 synthesis on a glass surface. Although we know of no description of the direct use of oligonucleotides as hybridisation probes while still attached to the matrix on which they were synthesised, there are reports of the use of oligonucleotides as hybridisation probes on

15 solid supports to which they were attached after synthesis. PCT Application WO 85/01051 describes a method for synthesising oligonucleotides tethered to a CPG column. In an experiment performed by us, CPG was used as the support in an Applied Bio-sytems

20 oligonucleotide synthesiser to synthesise a 13-mer complementary to the left hand cos site of phage lambda. The coupling steps were all close to theoretical yield. The first base was stably attached to the support medium through all the synthesis and

25 deprotection steps by a covalent link.

5.4 ANALYSING SEVERAL SEQUENCES SIMULTANEOUSLY

The method of this invention can be used to analyse several polynucleotide sequences

30 simultaneously. To achieve this, the oligonucleotides may be attached to the support in the form of (for example) horizontal stripes. A technique for doing this is described in Example 3 below. Each DNA sample to be analysed is labelled and applied to the surface

35 carrying the oligonucleotides in the form of a stripe (e.g. vertical) orthogonal to the oligonucleotide

stripes of the array. Hybridisation is seen at the
intersections between oligonucleotide stripes and
stripes of test sequence where there is homology
between them.

Where sequence variations are known, an
advantage of using this technique is that many
different mutations can be probed simultaneously by
laying down stripes corresponding to each allelic
variant. With a density of one oligonucleotide per
mm, and one "individual" per 5 mm, it should be
possible to analyse 2000 loci on a plate 100 mm square.
Such a high density of information, where the
oligonucleotides do identify specific alleles, is not
available by other techniques.

6. PROBES, HYBRIDISATION AND DETECTION

The yield of oligonucleotides synthesised on
microporous glass is about 30 µmol/g. A patch of this
material 1 micron thick by 10 microns square would hold
$\approx 3 \times 10^{-12}$ µmol, equivalent to about 2 g of human

DNA. The hybridisation reaction could therefore be
carried out with a very large excess of the bound
oligonucleotides over that in the probe. So it should
be possible to design a system capable of
5   distinguishing between hybridisation involving single
and multiple occurrances of the probe sequence, as
yield will be proportional to concentration at all
stages in the reaction.

The polynucleotide sequence to be analysed may be
10 of DNA or RNA. To prepare the probe, the polynucleotide
may be degraded to form fragments. Preferably it is
degraded by a method which is as random as possible, to
an average length around the chosen length s of the
oligonucleotides on the support, and oligomers of exact
15 length s selected by electrophoresis on a sequencing
gel. The probe is then labelled. For example,
oligonucleotides of length s may be end labelled. If
labelled with ^{32}P, the radioactive yield of any
individual s-mer even from total human DNA could be
20 more than 10^4 dpm/mg of total DNA. For detection, only
a small fraction of this is needed in a patch 10-100
microns square. This allows hybridisation conditions
to be chosen to be close to the Tm of duplexes, which
decreases the yield of hybrid and decreases the rate of
25 formation, but increases the discriminating power.
Since the bound oligonucleotide is in excess, signal
need not be a problem even working close to
equilibrium.

Hybridisation conditions can be chosen to be those
30 known to be suitable in standard procedures used to
hybridise to filters, but establishing optimum
conditions is important. In particular, temperature
needs to be controlled closely, preferably to better
than $\pm 0.5°C$. Particularly when the chosen length of
35 the oligonucleotide is small, the analysis needs to be

able to distinguish between slight differences of rate
and/or extent of hybridisation. The equipment may need
to be programmed for differences in base composition
between different oligonucleotides. In constructing
5   the array, it may be preferable to partition this into
sub-matrices with similar base compositions. This may
make it easier to define the Tm which may differ
slightly according to the base composition.

     The choice of hybridisation solvent is
10 significant. When 1M NaCl is used, G:C base pairs are
more stable than A:T base pairs. Double stranded
oligonucleotides with a high G+Ccontent have a higher
Tm than corresponding oligonucleotides with a high A+T
content. This discrepancy can be compensated in
15 various ways: the amount of oligonucleotide laid down
on the surface of the support can be varied depending
on its nucleotide composition; or the computer used to
analyse the data can be programmed to compensate for
variations in nucleotide composition. A preferred
20 method, which can be used either instead of or in
addition to those already mentioned, is to use a
chaotropic hybridisation solvent, for example a
quarternary or tertiary amine as mentioned above.
Tetramethylammoniumchloride (TMACl) has proved
25 particularly suitable, at concentrations in the range 2
M to 5.5 M. At TMACl concentrations around 3.5 M to 4
M, the T_m dependence on nucleotide composition is
greatly reduced.

     The nature of the hybridisation salt used also has
30 a major effect on the overall hybridisation yield.
Thus, the use of TMACl at concentrations up to 5 M can
increase the overall hybridisation yield by a factor of
30 or more (the exact figure depending to some extent
on nucleotide composition) in comparison with
35 hybridisation using 1M NaCl. Manifestly, this has
important implications; for example the amount of

probe material that needs to be used to achieve a given signal can be much lower.

Autoradiography, especially with ^{32}P causes image degradation which may be a limiting factor determining resolution; the limit for silver halide films is around 25 microns. Obviously some direct detection system would be better. Fluorescent probes are envisaged; given the high concentration of the target oligonucleotides, the low sensitivity of fluorescence may not be a problem.

We have considerable experience of scanning auto-radiographic images with a digitising scanner. Our present design is capable of resolution down to 25 microns, which could readily be extended down to less than present application, depending on the quality of the hybridisation reaction, and how good it is at distinguishing absence of a sequence from the presence of one or more. Devices for measuring astronomical plates have an accuracy around 1 μ. Scan speeds are such that a matrix of several million cells can be scanned in a few minutes. Software for the analysis of the data is straight-forward, though the large data sets need a fast computer.

Experiments presented below demonstrate the feasibility of the claims.

Commercially available microscope slides (BDH Super Premium 76 x 26 x 1 mm) were used as supports. These were derivatised with a long aliphatic linker that can withstand the conditions used for the

deprotection of the aromatic heterocyclic bases, i.e.
30% NH_3 at 55° for 10 hours. The linker, bearing a
hydroxyl group which serves as a starting point for the
subsequent oligonucleotide, is synthesised in two
steps. The slides are first treated with a 25%
solution of 3-glycidoxypropyltriethoxysilane in xylene
containing several drops of Hunig's base as a catalyst.
The reaction is carried out in a staining jar, fitted
with a drying tube, for 20 hours at 90^oC. The slides
are washed with MeOH, Et_2O and air dried. Then neat
hexaethylene glycol and a trace amount of conc.
sulphuric acid are added and the mixture kept at 80°
for 20 hours. The slides are washed with MeOH, Et_2O ,
air dried and stored desiccated at -20° until use.
This preparative technique is described in British
Patent Application 8822228.6 filed 21 September 1988.

The oligonucleotide synthesis cycle is performed
as follows:

The coupling solution is made up fresh for each
step by mixing 6 vol. of 0.5M tetrazole in anhydrous
acetonitrile with 5 vol. of a 0.2M solution of the
required beta-cyanoethylphosphoramidite. Coupling time
is three minutes. Oxidation with a 0.1M solution
of I_2 in THF/pyridine/H_2O yields a stable phospho-
triester bond. Detritylation of the 5' end with 3%
trichloroacetic acid in dichloromethane allows further
extension of the oligonucleotide chain. There was no
capping step since the excess of phosphoramidites used
over reactive sites on the slide was large enough to
drive the coupling to completion. After the synthesis
is completed, the oligonucleotide is deprotected in 30%
NH_3 for 10 hours at 55°. The chemicals used in the
coupling step are moisture-sensitive, and this critical
step must be performed under anhydrous conditions in a
sealed container, as follows. The shape of the patch

to be synthesised was cut out of a sheet of silicone
rubber (76 x 26 x 0.5 mm) which was sandwiched between
a microscope slide, derivatised as described above, and
a piece of teflon of the same size and thickness.   To
5   this was fitted a short piece of plastic tubing that
allowed us to inject and withdraw the coupling solution
by syringe and to flush the cavity with Argon.   The
whole assembly was held together by fold-back paper
clips.   After coupling the set-up was disassembled and
10   the slide put through the subsequent chemical reactions
(oxidation with iodine, and detritylation by treatment
with TCA) by dipping it into staining jars.

EXAMPLE 1.

As a first example we synthesised the sequences
15   oligo-dT$_{10}$-oligo-dT$_{14}$ on a slide by gradually decreasing
the level of the coupling solution in steps 10 to 14.
Thus the 10-mer was synthesised on the upper part of
the slide, the 14-mer at the bottom and the 11, 12 and
13-mers were in between.   We used 10 pmol oligo-dA$_{12}$,
20   labelled at the 5' end with ^{32}P by the polynucleotide
kinase reaction to a total activity of 1.5 million
c.p.m., as a hybridisation probe.   Hybridisation was
carried out in a perspex (Plexiglas) container made to
fit a microscope slide, filled with 1.2 ml of 1M NaCl
25   in TE, 0.1% SDS, for 5 minutes at 20^0.   After a short
rinse in the same solution without oligonucleotide, we
were able to detect more than 2000 c.p.s. with a
radiation monitor.   An autoradiograph showed that all
the counts came from the area where the oligonucleotide
30   had been synthesised, i.e. there was no non-specific
binding to the glass or to the region that had been
derivatised with the linker only.   After partial
elution in 0.1 M NaCl differential binding to the
target is detectable, i.e. less binding to the shorter
35   than the longer oligo-dT.   By gradually heating the

slide in the wash solution we determined the T_{om} (mid-point of transition when 50% eluted) to be 33°. There were no counts detectable after incubation at 39°. The hybridisation and melting was repeated eight times with no diminution of the signal. The result is reproducible. We estimate that at least 5% of the input counts were taken up by the slide at each cycle.

EXAMPLE 2.

In order to determine whether we would be able to distinguish between matched and mismatched oligo-nucleotides we synthesised two sequences 3' CCC GCC GCT GGA (cosL) and 3' CCC GCC TCT GGA, which differ by one base at position 7. All bases except the seventh were added in a rectangular patch. At the seventh base, half of the rectangle was exposed in turn to add the two different bases, in two stripes. Hybridisation of cosR probe oligonucleotide (5' GGG CGG CGA CCT) (kinase labelled with ^{32}P to 1.1 million c.p.m., 0.1 M NaCl, TE, 0.1% SDS) was for 5 hours at 32°. The front of the slide showed 100 c.p.s. after rinsing. Autoradiography showed that annealing occurred only to the part of the slide with the fully complementary oligonucleotide. No signal was detectable on the patch with the mismatched sequence.

EXAMPLE 3.

For a further study of the effects of mismatches or shorter sequences on hybridisation behaviour, we constructed two arrays; one (a) of 24 oligonucleotides and the other (b) of 72 oligonucleotides.

These arrays were set out as shown in Table 1(a) and 1(b). The masks used to lay down these arrays were different from those used in previous experiments. Lengths of silicone rubber tubing (1mm o.d.) were glued with silicone rubber cement to the surface of plain microscope slides, in the form of a "U". Clamping

these masks against a derivatised microscope slide
produced a cavity into which the coupling solution was
introduced through a syringe. In this way only the
part of the slide within the cavity came into contact
with the phosphoramidite solution. Except in the
positons of the mismatched bases, the arrays listed in
Table 1 were laid down using a mask which covered most
of the width of the slide. Off-setting this mask by
3mm up or down the derivatised slide in subsequent
coupling reactions produced the olignucleotides
truncated at the 3' or 5' ends.

For the introduction of mismatches a mask was used
which covered half (for array (a)) or one third (for
array (b)) of the width of the first mask. The bases
at positions six and seven were laid down in two or
three longitudinal stripes. This led to the synthesis
of oligonucleotides differing by one base on each half
(array (a)) or third (array (b)) of the slide. In
other positions, the sequences differed from the
longest sequence by the absence of bases at the ends.

In array (b), there were two columns of sequences
between those shown in Table 1(b), in which the sixth
and seventh bases were missing in all positions,
because the slide was masked in a stripe by the
silicone rubber seal. Thus there were a total of 72
different sequences represented on the slide in 90
different positions.

The 19-mer 5' CTC CTG AGG AGA AGT CTG C was used
for hybridisation (2 million cpm, 1.2 ml 0.1M NaCl in
TE, 0.1% SDS, 20°).

The washing and elution steps were followed by
autoradiography. The slide was kept in the washing
solution for 5 min at each elution step and then
exposed (45 min, intensified). Elution temperatures
were 23, 36, 42, 47, 55 and 60°C respectively.

As indicated in the table, the oligonucleotides
showed different melting behaviour.   Short oligo-
nucleotides melted before longer ones, and at 55°C,
only the perfectly matched 19-mer was stable, all other
oligonucleotides had been eluted.   Thus the method can
differentiate between a 18-mer and a 19-mer which
differ only by the absence of one base at the end.
Mismatches at the end of the oligonucleotides and at
internal sites can all be melted under conditions where
the perfect duplex remains.

Thus we are able to use very stringent
hybridisation conditions that eliminate annealing to
mismatch sequences or to oligonucleotides differing in
length by as little as one base.   No other method using
hybridisation of oligonucleotides bound to the solid
supports is so sensitive to the effects of mismatching.

EXAMPLE 4.

To test the application of the invention to
diagnosis of inherited diseases, we hybridised the
array (a), which carries the oligonucleotide sequences
specific for the wild type and the sickle cell mutations
of the β -globin gene, with a 110 base pair fragment of
DNA amplified from the β -globin gene by means of the
polymerase chain reaction (PCR).   Total DNA from the
blood of a normal individual (1 microgram) was
amplified by PCR in the presence of appropriate primer
oligonucleotides.   The resulting 110 base pair fragment
was purified by electrophoresis through an agarose gel.
After elution, a small sample (ca. 10 picogram) was
labelled by using α - ^{32}P-dCTP (50 microCurie) in a
second PCR reaction.   This PCR contained only the
upstream priming oligonucleotide.   After 60 cycles of
amplification with an extension time of 9 min. the
product was removed from precursors by gel filtration.
Gel electrophoresis of the radioactive product showed a

major band corresponding in length to the 110 base fragment. One quarter of this product (100,000 c.p.m. in 0.9 M NaCl, TE, 0.1% SDS) was hybridised to the array (a). After 2 hours at 30°ca. 15000 c.p.m. had been taken up. The melting behaviour of the hybrids was followed as described for the 19-mer in example 3, and it was found that the melting behaviour was similar to that of the oligonucleotide. That is to say, the mismatches considerably reduced the melting temperature of the hybrids, and conditions were readily found such that the perfectly matched duplex remained whereas the mismatched duplexes had fully melted.

Thus the invention can be used to analyse long fragments of DNA as well oligonucleotides, and this example shows how it may be used to test nucleic acid sequences for mutations. In particular it shows how it may be applied to the diagnosis of genetic diseases.

EXAMPLE 5.

To test an automated system for laying down the precursors, the ccsL oligonucleotide was synthesised with 11 of the 12 bases added in the way described above. For the addition of the seventh base, however, the slide was transferred into an Argon filled chamber containing a pen plotter. The pen of the plotter had been replaced by a component, fabricated from Nylon, which had the same shape and dimensions as the pen, but which carried a polytetrafluoroethylene (PTFE) tube, through which chemicals could be delivered to the surface of the glass slide which lay on the bed of the plotter. A microcomputer was used to control the plotter and the syringe pump which delivered the chemicals. The pen, carrying the delivery tube from the syringe, was moved into position above the slide, the pen was lowered and the pump activated to lay down coupling solution. Filling the pen successively with

G, T and A phosphoramidite solutions an array of twelve
spots was laid down in three groups of four, with three
different oligonucleotide sequences.   After
hybridisation to cosR, as described in Example 2, and
5   autoradiography, signal was seen only over the four
spots of perfectly matched oligonucleotides, where the
dG had been added.

EXAMPLE 6

This example demonstrates the technique of
10   analysing several DNA sequences simultaneously.   Using
the technique described in Example 3, a slide was
prepared bearing six parallel rows of oligonucleotides
running along its length.   These comprised duplicate
hexadecamer sequences corresponding to antisense
15   sequences of the β-globin wild-type (A), sickle cell ·
(S) and C mutations.

Clinical samples of AC, AS and SS DNA were
procured.   Three different single-stranded probes of
110 nt length with approx. 70,000 c.p.m. in 100 µl 1M
20   NaCl, TE pH 7.5, 0.1% SDS, viz AC, AS, and SS DNA were
prepared.   Radiolabelled nucleotide was included in the
standard PCR step yielding a double-stranded labelled
fragment.   It was made single-stranded with
Bacteriophage λ exonuclease that allowed to selectively
25   digest one strand bearing a 5´ phosphate.   This was
made possible by phosphorylating the downstream primer
with T4 Polynucleotide kinase and (´cold´) ATP prior to
PCR.   These three probes were applied as three stripes
orthogonal to the surface carrying the six
30   oligonucleotide stripes.   Incubation was at 30°C for 2
hours in a moist chamber.   The slide was then rinsed at
ambient temperature, then 45°C for 5 minutes and
exposed for 4 days with intensification.   The genotype
of each clinical sample was readily determined from the
35   autoradiographic signals at the points of intersection.

EXAMPLE 7

A plate was prepared whose surface carried an array of all 256 octapurines. That is to say, the array comprised 256 oligonucleotides each consisting of a different sequence of A and G nucleotides. This array was probed with a mixture comprising all 256 octapyrimidines, each end labelled by means of polynucleotide kinase and γ-^{32}P-ATP. Hybridisation was performed for 6 - 8 hours at 4°C.

In consecutive experiments the hybridisation solvent was changed through the series 1M NaCl (containing 10mM Tris.HCl pH 7.5, 1mM EDTA, 7% sarcosine) and 2M, 2.5M, 3M, 3.5M, 4M, 4.5M, 5M and 5.5M TMACl (all containing 50mM Tris.HCl pH 8.0, 2mM EDTA, SDS at less than 0.04 mg/ml). The plate was rinsed for 10 minutes at 4°C in the respective solvent to remove only loosely matched molecules, sealed in a plastic bag and exposed to a PhorphorImager storage phosphor screen at 4°C overnight in the dark.

The following table quotes relative signal intensities, at a given salt concentration, of hybrids formed with oligonucleotides of varying a content. In this table, the first row refers to the oligonucleotide GGGGGGGG, and the last row to the oligonucleotide AAAAAAAA. It can be seen that the difference in response of these two oligonucleotides is marked in 1M NaCl, but much less marked in 3M or 4M TMACl.

Relative Intensities at given Salt Concentration

Solvent	Number of A's		
	0	4	8
1M NaCl	100	30	20
2M TMACl	100	70	30
3M TMACl	70	100	40
4M TMACl	60	100	40

The following table indicates relative signal intensities obtained, with octamers containing 4A's and 4G's, at different hybridisation salt concentrations. It can be seen that the signal intensity is dramatically increased at higher concentrations of TMACl.

Relative Intensities at different Salt Concentrations

Solvent	Yield of hybrid
1M NaCl	100
2M TMACl	200
3M TMACl	700
4M TMACl	2000

In conclusion, we have demonstrated the following:
1. It is possible to synthesise oligonucleotides in good yield on a flat glass plate.

2.   Multiple sequences can be synthesised on the sample
in small spots, at high density, by a simple manual
procedure, or automatically using a computer controlled
device.

5   3.   Hybridisation to the oligonucleotides on the plate
can be carried out by a very simple procedure.
Hybridisation is efficient, and hybrids can be detected
by a short autoradiographic exposure.

4.   Hybridisation is specific. There is no detectable

10   signal on areas of the plate where there are no
oligonucleotides.   We have tested the effects of mis-
matched bases, and found that a single mismatched base
at any position in oligonucleotides ranging in length
from 12-mer to 19-mer reduces the stability of the

15   hybrid sufficiently that the signal can be reduced to a
very low level, while retaining significant
hybridisation to the perfectly matched hybrid.

5.   The oligonucleotides are stably bound to the glass
and plates can be used for hybridisation repeatedly.

20   The invention thus provides a novel way of
analysing nucleotide sequences, which should find a
wide range of application. We list a number of
potential applications below:

25

30

35

Small arrays of oligonucleotides as fingerprinting and mapping tools

Analysis of known mutations including genetic diseases.

Example 4 above shows how the invention may be
5   used to analyse mutations.   There are many
applications for such a method, including the detection
of inherited diseases.

Genomic fingerprinting.

In the same way as mutations which lead to disease
10 can be detected, the method could be used to detect
point mutations in any stretch of DNA.   Sequences are
now available for a number of regions containing the
base differences which lead to restriction fragment
length polymorphisms (RFLPs).   An array of oligo-
15 nucleotides representing such polymorphisms could be
made from pairs of oligonucleotides representing the
two allelic restriction sites.   Amplification of the
sequence containing the RFLP, followed by hybridisation
to the plate, would show which alleles were present in
20 the test genome.   The number of oligonucleotides that
could be analysed in a single analysis could be quite
large.   Fifty pairs made from selected alleles would
be enough to give a fingerprint unique to an individual.

Linkage analysis.

25 Applying the method described in the last
paragraph to a pedigree would pinpoint recombinations.
Each pair of spots in the array would give the
information that is seen in the track of the RFLP
analysis, using gel electrophoresis and blotting, that
30 is now routinely used for linkage studies.   It should
be possible to analyse many alleles in a single
analysis, by hybridisation to an array of allelic pairs
of oligonucleotides, greatly simplifying the methods
used to find linkage between a DNA polymorphism and
35 phenotypic marker such as a disease gene.

The examples above could be carried out using the

method we have developed and confirmed by experiments.

Large arrays of oligonucleotides as sequence reading tools.

We have shown that oligonucleotides can be synthesised in small patches in precisely determined positions by one of two methods: by delivering the precursors through the pen of a pen-plotter, or by masking areas with silicone rubber.   It is obvious how a pen plotter could be adapted to synthesise large arrays with a different sequence in each position. For some applications the array should be a predetermined, limited set; for other applications, the array should comprise every sequence of a predetermined length. The masking method can be used for the latter by laying down the precursors in a mask which produces intersecting lines.   There are many ways in which this can be done and we give one example for illustration:

1.   The first four bases, A, C, G, T, are laid in four broad stripes on a square plate.

2.   The second set is laid down in four stripes equal in width to the first, and orthogonal to them.   The array is now composed of all sixteen dinucleotides.

3.   The third and fourth layers are laid down in four sets of four stripes one quarter the width of the first stripes.   Each set of four narrow stripes runs within one of the broader stripes. The array is now composed of all 256 tetranucleotides.

4.   The process is repeated, each time laying down two layers with stripes which are one quarter the width of the previous two layers.   Each layer added increases the length of the oligonucleotides by one base, and the number of different oligonucleotide sequences by a factor of four.

The dimensions of such arrays are determined by the width of the stripes.   The narrowest stripe we

have laid is 1mm, but this is clearly not the lowest limit.

There are useful applications for arrays in which part of the sequence is predetermined and part made up of all possible sequences. For example:

Characterising mRNA populations.

Most mRNAs in higher eukaryotes have the sequence AAUAAA close to the 3' end. The array used to analyse mRNAs would have this sequence all over the plate. To analyse a mRNA population it would be hybridised to an array composed of all sequences of the type $N_m AATAAAN_n$. For m + n = 8, which should be enough to give a unique oligonucleotide address to most of the several thousand mRNAs that is estimated to be present in a source such as a mammalian cell, the array would be 256 elements square. The 256 x 256 elements would be laid on the AATAAA using the masking method described above. With stripes of around 1mm, the array would be ca. 256mm square.

This analysis would measure the complexity of the mRNA population and could be used as a basis for comparing populations from different cell types. The advantage of this approach is that the differences in the hybridisation pattern would provide the sequence of oligonucleotides that could be used as probes to isolate all the mRNAs that differed in the populations.

Sequence determination.

To extend the idea to determine unkown sequences, using an array composed of all possible oligonucleotides of a chosen length, requires larger arrays than we have synthesised to date. However, it is possible to scale down the size of spot and scale up the numbers to those required by extending the methods we have developed and tested on small arrays. Our experience shows that the method is much simpler in operation than the gel based methods.

TABLE 1

For Examples 3 and 4 array (a) was set out as follows:

```
    20  GAG GAC TCC TCT ACG            20  GAG GAC aCC TCT ACG
    36  GAG GAC TCC TCT GAC G          20  GAC GAC aCC TCT GAC G
    36  GAG GAC TCC TCT AGA CG         20  GAC GAC aCC TCT AGA CG
    47  GAG GAC TCC TCT CAG ACG        36  GAG GAC aCC TCT CAG ACG
  5 60  GAG GAC TCC TCT TCA GAC G      47  GAG GAC aCC TCT TCA GAC G
    56  .AG GAC TCC TCT TCA GAC G      42  .AG GAC aCC TCT TCA GAC G
    56  ..G GAC TCC TCT TCA GAC G      42  ..G GAC aCC TCT TCA GAC G
    47  ... GAC TCC TCT TCA GAC G      42  ... GAC aCC TCT TCA GAC G
    42  ... .AC TCC TCT TCA GAC G      36  ... .AC aCC TCT TCA GAC G
 10 36  ... ..C TCC TCT TCA GAC G      36  ... ..C aCC TCT TCA GAC G
    36  ... ... TCC TCT TCA GAC G      36  ... ... aCC TCT TCA GAC G
    36  ... ... .CC TCT TCA GAC G      36  ... ... .CC TCT TCA GAC G
```

For example 3 array (b) was set out as follows:

```
 15 20 GAG GAt TC          20 GAG GAC TC          20 GAG GAC aC
    20 GAG GAt TCC         20 GAG GAC TCC         20 GAG GAC aCC
    20 GAG GAt TCC T       20 GAG GAC TCC T       20 GAG GAC aCC T
    20 GAG GAt TCC TC      20 GAG GAC TCC TC      20 GAG GAC aCC TC
    20 GAG GAt TCC TCT     20 GAG GAC TCC TCT     20 GAG GAC aCC TCT
 20 20 GAG GAt TCC TCT T   20 GAG GAC TCC TCT T   20 GAG GAC aCC TCT T
    20 GAG GAt TCC TCT TC  20 GAG GAC TCC TCT TC  20 GAG GAC aCC TCT TC
    20 GAG GAt TCC TCT TCA 20 GAG GAC TCC TCT TCA 20 GAG GAC aCC TCT TCA
    32 GAG GAt TCC TCT TCA G    42 GAG GAC TCC TCT TCA G    20 GAG GAC aCC TCT TCA G
    32 GAG GAt TCC TCT TCA GA   47 GAG GAC TCC TCT TCA GA   32 GAG GAC aCC TCT TCA GA
 25 42 GAG GAt TCC TCT TCA GAC  52 GAG GAC TCC TCT TCA GAC  42 GAG GAC aCC TCT TCA GAC
    52 GAG GAt TCC TCT TCA GAC G 60 GAG GAC TCC TCT TCA GAC G 52 GAG GAC aCC TCT TCA GAC G
    42 .AG GAt TCC TCT TCA GAC G  52 .AG GAC TCC TCT TCA GAC G  42 .AG GAC aCC TCT TCA GAC G
    42 ..G GAt TCC TCT TCA GAC G  52 ..G GAC TCC TCT TCA GAC G  42 ..G GAC aCC TCT TCA GAC G
    37 ... GAt TCC TCT TCA GAC G  47 ... GAC TCC TCT TCA GAC G  37 ... GAC aCC TCT TCA GAC G
 30 32 ... .At TCC TCT TCA GAC G  42 ... .AC TCC TCT TCA GAC G  32 ... .AC aCC TCT TCA GAC G
    32 ... ..t TCC TCT TCA GAC G  42 ... ..C TCC TCT TCA GAC G  32 ... ..C aCC TCT TCA GAC G
    32 ... ... TCC TCT TCA GAC G  32 ... ... TCC TCT TCA GAC G  32 ... ... aCC TCT TCA GAC G
```

Between the three columns of array (b) listed above, were two
columns, in which bases 6 and 7 from the left were missing in
35 every line. These sequences all melted at 20 or 32 degrees.
(a,t) mismatch base (.) missing base.

CLAIMS

1.   Apparatus for analysing a polynucleotide sequence,
comprising a support and attached to a surface thereof an array of
the whole or a chosen part of a complete set of oligonucleotides of
chosen lengths, the different oligonucleotides occupying separate
cells of the array and being capable of taking part in
hybridisation reactions.

2.   Apparatus for studying differences between polynucleotide
sequences, comprising a support and attached to a surface thereof
an array of the whole or a chosen part of a complete set of
oligonucleotides of chosen lengths comprising the polynucleotide
sequences, the different oligonucleotides occupying separate cells
of the array and being capable of taking part in hybridisation
reactions.

3.   Apparatus as claimed in claim 2, wherein the array
comprises one or more pairs of oligonucleotides of chosen lengths.

4.   Apparatus as claimed in claim 3, wherein the array
comprises one or more pairs of oligonucleotides of chosen lengths
representing normal and mutant versions of a point mutation to be
studied.

5.   Apparatus as claimed in any one of claims 1 or 2, wherein
the chosen length is from 8 to 20 nucleotides.

6.   Apparatus as claimed in any one of claims 1 or 2, wherein
the surface of the support to which the oligonucleotides are
attached is of glass.

7.   Apparatus as claimed in any one of claims 1 or 2 , wherein
each oligonucleotide is bound to the support through a covalent
link.

8.   A method of analysing a polynucleotide sequence, by the
use of a support to the surface of which is attached an array of
the whole or a chosen part of a complete set of oligonucleotides of
chosen lengths, the different oligonucleotides occupying separate
cells of the array, which method comprises labelling the
polynucleotide sequence or fragments thereof to form labelled
material,

applying the labelled material under hybridisation conditions to the array, and observing the location of the label on the surface associated with particular members of the set of oligonucleotides.

9. A method according to claim 8, applied to the study of differences between polynucleotide sequences, wherein the array is of the whole or a chosen part of the complete set of oligonucleotides of chosen lengths comprising the polynucleotide sequences.

10. A method as claimed in claim 9, wherein the array comprises one or more pairs of oligonucleotides of chosen lengths.

11. A method as claimed in claim 10, wherein the array comprises one or more pairs of oligonucleotides of chosen lengths representing normal and mutant versions of a point mutation being studied.

12. A method according to any one of claims 8 to 11, wherein the polynucleotide sequence is randomly degraded to form a mixture of oligomers of a chosen length, the mixture being thereafter labelled to form the labelled material.

13. A method as claimed in claim 12, wherein the oligomers are labelled with ^{32}P.

14. A method as claimed in any one of claims 8 to 11, wherein the chosen length is from 8 to 20 nucleotides.

15. A method as claimed in claim 8, where the set of oligonucleotides is attached to the surface as an array of parallel stripes, and at least two polynucleotide sequences are analysed simultaneously by applying the labelled material to the array in the form of separate stripes orthogonal to the oligonucleotide stripes.

16. A method as claimed in claim 8, wherein hybridisation is effected in the presence of tetramethylammoniumchloride at a concentration of 1M to 5M.

ABSTRACT

This invention provides an apparatus and method for analyzing a polynucletoide sequence; either an unknown sequence or a known sequence. A support, e.g. a glass plate, carries an array of the whole or a chosen part of a complete set of oligonucletoides which are capable of taking part in hybridization reactions. The array may comprise one or more pair of oligonucleotides of chosen lengths. The polynucleotide sequence, or fragments thereof, are labelled and applied to the array under hybridizing conditions. Applications include analyses of known point mutations, genomic fingerprinting, linkage analysis, characterization of mRNAs, mRNA populations, and sequence determination.

DECLARATION AND POWER OF ATTORNEY FOR U.S PATENT APPLICATIONS

(X) Original ( ) Supplemental ( ) Substitute ( ) PCT ( ) DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: _____ ANALYSING POLYNUCLEOTIDE SEQUENCES _____

_____

which is described and claimed in:
( ) the attached specification, or
(X) the specification in the application Serial No. __07/695,682__ filed __May 3, 1991__ ;
and with amendments through _____ (if applicable),
( ) the specification in International Application No. PCT/ _____ , filed
_____ , and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED	
Great Britain	8810400.5	May 3, 1988	(X) YES	( ) NO
			( ) YES	( ) NO
			( ) YES	( ) NO
			( ) YES	( ) NO
			( ) YES	( ) NO
			( ) YES	( ) NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

SERIAL NO.	U.S. FILING DATE	STATUS		
PCT/GB89/00460	May 2, 1989	( ) Patented (X) Pending ( ) Abandoned		
07/573,317	September 28, 1990	( ) Patented (X) Pending ( ) Abandoned		
		( ) Patented ( ) Pending ( ) Abandoned		

And I hereby appoint V. M. Creedon, Reg. No. 17111, John T. Miller, Reg. No. 21120, John T. Fedigan, Reg. No. 24347, Michael R. Davis, Reg. No. 25134, Matthew M. Jacob, Reg. No. 25154, Jeffrey Nolton, Reg. No. 25408, and Henry M. Zykorie, Reg. No. 27477, who together constitute the firm of WENDEROTH, LIND & PONACK, jointly and severally, attorneys to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from _____
STEVENS, HEWLETT & PERKINS _____ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

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I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor _Edwin South_ Date _20th July 1991_
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3rd Inventor _____ Date _____

4th Inventor _____ Date _____

5th Inventor _____ Date _____

6th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U. S. Application Serial No. 07/695,682 , Filing Date May 3, 1991

Applicant Reference Number PP/IR/7644/USCIP, Atty Docket No. 263/PPIR7644USCIP

Title of Invention ANALYSING POLYNUCLEOTIDE SEQUENCES